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TITLE: Design and Development of Peptides from the Anti-Angiogenic Pigment Epithelial-Derived Factor for the Therapy of Prostate Cancer

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14. ABSTRACT
To create PEDF based therapy for hormone-refractory CaP we have proposed to design short synthetic peptides corresponding to the 34-mer anti-angiogenic epitope of PEDF. The 3D structure of PEDF 34-mer peptide was analyzed using Protean software in terms of relative hydrophobicity, charge distribution, and antigenic index. Three synthetic peptides covering the 34-mer PEDF fragment were generated and tested for the ability to reproduce anti-angiogenic effect of PEDF. Although all peptides (14, 18 and 23-mer) inhibited FGF-induced endothelial cell migration only 18 and 23-mer induced apoptosis in endothelial cells. The 18-mer peptide also blocked neovascularization induced by FGF in vivo as demonstrated in corneal and matrigel assays. This peptide was further tested in vivo in mouse model for ability to inhibit prostate tumor growth. Subcutaneous PC3 tumor growth was inhibited in mice treated with 18-mer peptide. There was significant reduction in microvascular density in the 18-mer treated animals accompanied by increased apoptosis.

15. SUBJECT TERMS
PEDF, anti-angiogenic inhibitor, peptides

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INTRODUCTION:

Pigment epithelial-derived factor (PEDF) is a potent natural angiogenesis inhibitor. We have previously mapped PEDF anti-angiogenic activity to its 34-mer N terminal epitope (residues 24-57) (1). This peptide retains PEDF anti-angiogenic properties: it reproduces signaling events in the endothelial cell (EC), elicits EC apoptosis and blocks migration. Forced expression of the 34-mer peptide delays the onset of prostate carcinoma. We proposed to further narrow down active anti-angiogenic epitope in order to create PEDF-based therapy for hormone-refractory CaP. We proposed to generate short synthetic peptides corresponding to the 34-mer epitope of PEDF and screen them for the ability to reproduce PEDF anti-angiogenic activity. In this study we evaluated and compared anti-angiogenic potential of the 34-mer peptide and its internal fragments *in vitro* by their ability to inhibit migration and to induce apoptosis in cultured endothelial cells. We further investigated biologically active peptides for the ability to block neovascularization *in vivo*. The most active compound, which retained high specific activity *in vivo*, was subjected for preclinical testing in mouse model of the hormone refractory prostate carcinoma.

BODY:

As indicated in the Statement of Work (SOW) during this 2-year period our task was to evaluate angiosuppresive activity of the short peptides covering anti-angiogenic 34-mer fragment of PEDF. Specific aims of this project were: 1) to identify peptide(s) with highest anti-angiogenic activity *in vitro*, which remain active *in vivo*; 2) to test their ability to inhibit prostate cancer growth in mouse model. In compliance with the SOW, in the course of the first year I generated series of short peptides covering anti-angiogenic 34-mer fragment and thoroughly evaluated their *in vitro* activities. The results were provided in the 2006 annual report (*Appendix 1*) and presented at the symposium on "Molecular Targets and Cancer Therapeutics" (*2*). Briefly, I have generated three shorter peptides (14-, 18-, and 23-mer) covering C-terminus of the 34-mer and screened them *in vitro* for the ability to inhibit EC migration and induce EC apoptosis. I have generated dose response curves for each of these in migration assay, determined and compared ED_{50} for each one. Based on this *in vitro* screening, the 18-mer was selected as the most efficient for further experiments. The ability of the peptides to block neovascularization *in vivo* was confirmed (a) in mouse corneal assay (b) in the matrigel plug assay and (c) in the Directed in Vivo Angiogenesis Assay (DIVAA, Trevigen, Inc.). Due to several problems that I encountered during the in vitro screening (described in details in the 2006 annual report) some of the experiments (particularly Matrigel plug assay) were delayed and were accomplished during the second year of the study. As indicated by the results of the first year, the 18-mer demonstrated better activity and less toxicity *in vitro* as well as *in vivo*, and was recommended for further testing in mouse model.

During the second year period I have performed additional DIVAA to compare activity of the short peptides with intact PEDF and parental 34-mer: this comparison confirmed the superb efficacy of the18-mer peptide *in vivo*. Furthermore, efficacy of the 18-mer was evaluated at two different doses in Matrigel plug assay. Lastly, this peptide was tested for the ability to inhibit the onset of prostate cancer in mouse xenograft model. The ability of the 18-mer to block tumor angiogenesis was also evaluated by the decrease in microvascular density (MVD) and apoptotic index of the tumors as was projected in SOW.

The following steps were accomplished during the final 2nd year:

1. I compared anti-angiogenic activity of all three peptides in DIVAA assay.
As described previously in the 1st annual report (Appendix 1), this assay allows to conserve the amount of peptide for testing, while giving quantitative evaluation of the anti-angiogenic activity (*3-5*). Using DIVAA, I confirmed that both the 34-mer and 18-mer retained anti-angiogenic activity *in vivo*, which was comparable to that of parental PEDF. DIVAA reactors (small silicon cylinders) containing basement membrane extract (BME), and/or pro-angiogenic factors (FGF/VEGF) in the presence or absence of PEDF peptides (1μM) were implanted subcutaneously in the flanks of nude mice (Fig.1 A). At day 12 post-implantation the reactors were excised and processed as previously described (*3, 5*). Endothelial cells were recovered from each reactor, labeled with FITC-lectin and fluorescence was measured in fluorimetric plate reader. The amount of fluorescence reflects the number of ECs that invaded angioreactors and therefore angiogenic response. Three independent experiments were performed, the results of each independent experiment were normalized per negative control and angiogenic activity was expressed as relative EC invasion (Fig. 1D).

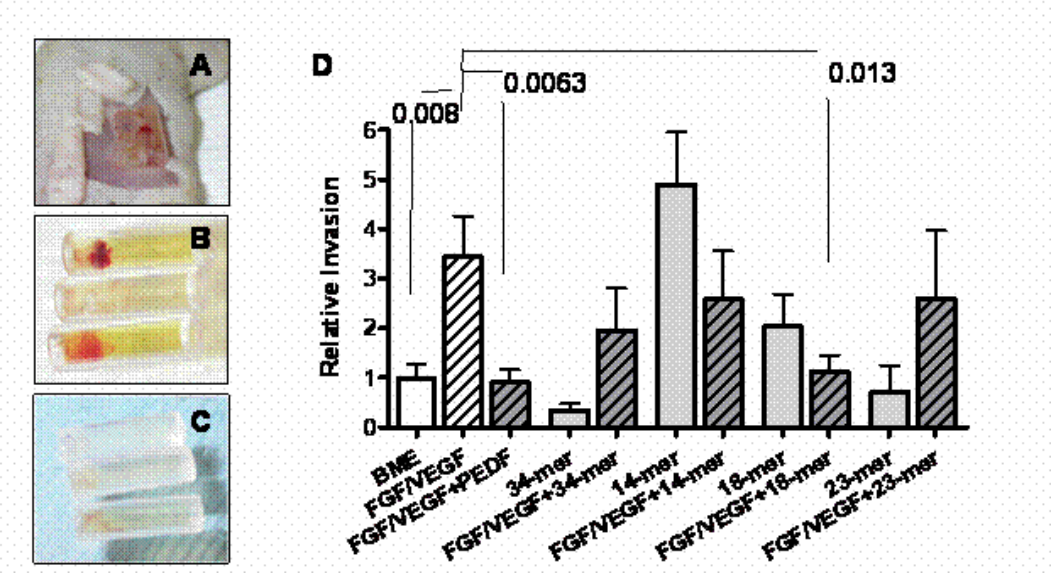

Figure1. PEDF peptides blocked FGF/VEGF induced neovascularization.
A. Angioreactors in situ; **B**. Extracted angioreactors with FGF/VEGF (37.5ng/12.5ng/reactor); **C**. Negative control, BME; **D**. Relative EC invasion.
Results of three independent experiments (minimum 4 reactors per condition were run in each experiment, n=4-31) are expressed as Mean ± SEM. Statistical significance was evaluated using student's t- test.

FGF/VEGF stimulated angiogenesis and caused 3 fold increases in vascularization compared to negative control (Fig. 1D). Addition of the 18-mer, 34-mer or PEDF significantly blocked angiogenesis ($p<0.01$). PEDF was the most effective, reducing vascularization to the level of negative control (BME). The 18-mer activity was comparable to that of PEDF and better than the activity of the 34-mer. In contrast, the 14-mer and 23-mer were unable to block angiogenesis *in vivo*. Interestingly, at tested concentration (1μM), 14-mer strongly promoted angiogenesis when tested alone with BME. DIVAA study helped me to identify the most promising anti-angiogenic peptide which I used in the following experiments for detailed comparison with the 34-mer.

2. In order to complete the first task outlined in SOW, I tested the 18-mer (2 doses) in the quantitative matrigel plug assay and compared its activity with the 34-mer. Matrigel plugs containing bFGF and the peptides were implanted in the median abdominal area of nude mice. I prepared cryosections of the matrigel plugs and stained them for the endothelial cell marker CD31 and apoptosis by TUNEL. Four mice were used per group per condition (40 mice total). Matrigel (BD Pharmingen) mixed with bFGF (250ng/ml), or bFGF + peptide at 10 and 100 nM was injected subcutaneously. Seven days later plugs were excised, snap-frozen, cryosections fixed and stained as described previously (1). Fluorescent images were obtained with Nikon epi-fluorescence microscope (Fig. 2)

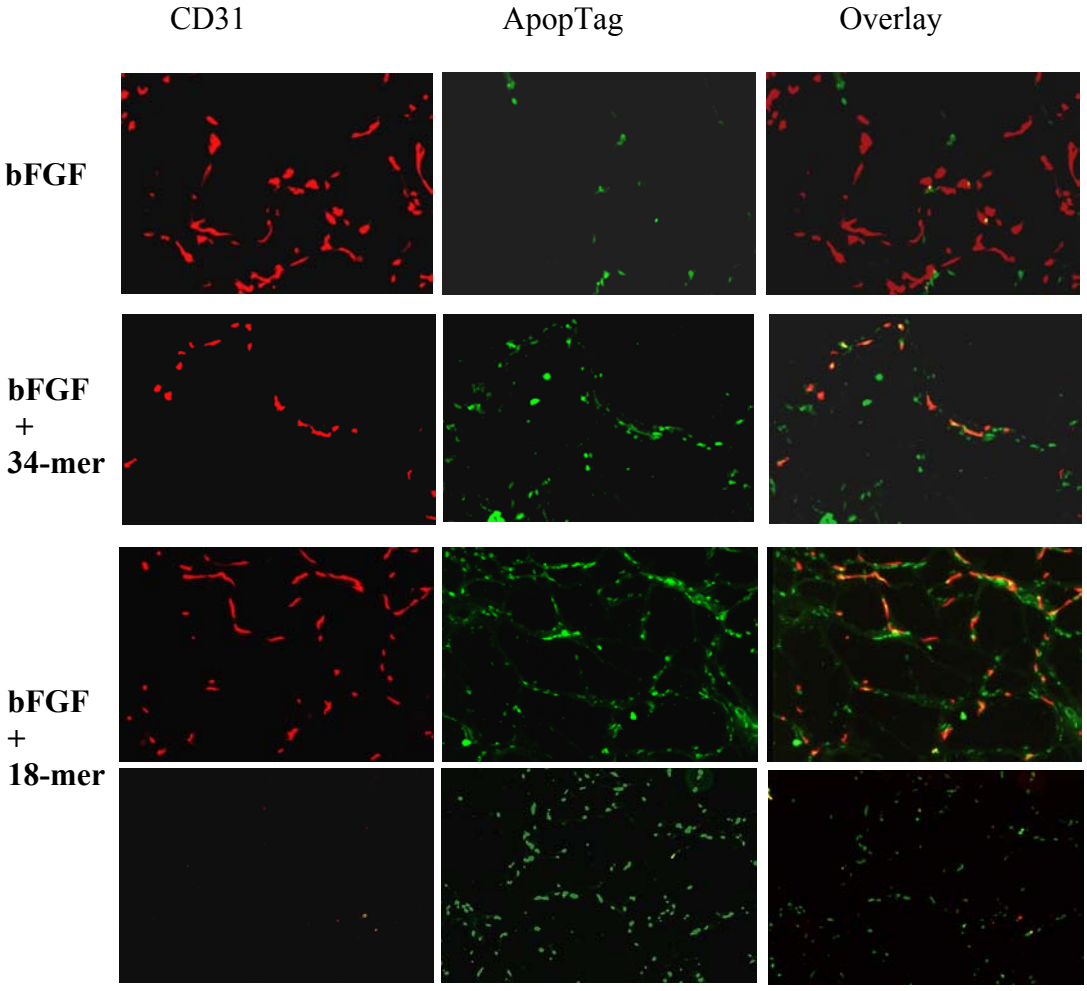

Figure2. PEDF-based peptides block in vivo vascularization by bFGF.
Matrigel plugs were stained with fluorescent-labeled antibody for the endothelial cell marker (CD31, red) and for apoptosis (ApopTag, TUNEL, green). Representative fields (X10 magnification) are shown. On the right, the overlay of the 2 fluorescent images is shown. Apoptotic cells appear yellow.

The efficacy of the peptide was determined as: (a) decrease in the MVD; (b) decrease in the vessels size (c) increase in the EC apoptosis. The plugs containing bFGF with either the 34-mer or the 18-mer showed less CD31 staining and more apoptosis (Fig.2). The overlay of the images revealed high co-localization of CD31 staining with apoptosis, suggesting that the

reduction in MVD is caused by EC apoptosis. The images were further quantified using MetaMorph software (Molecular Devices) (Fig. 3).

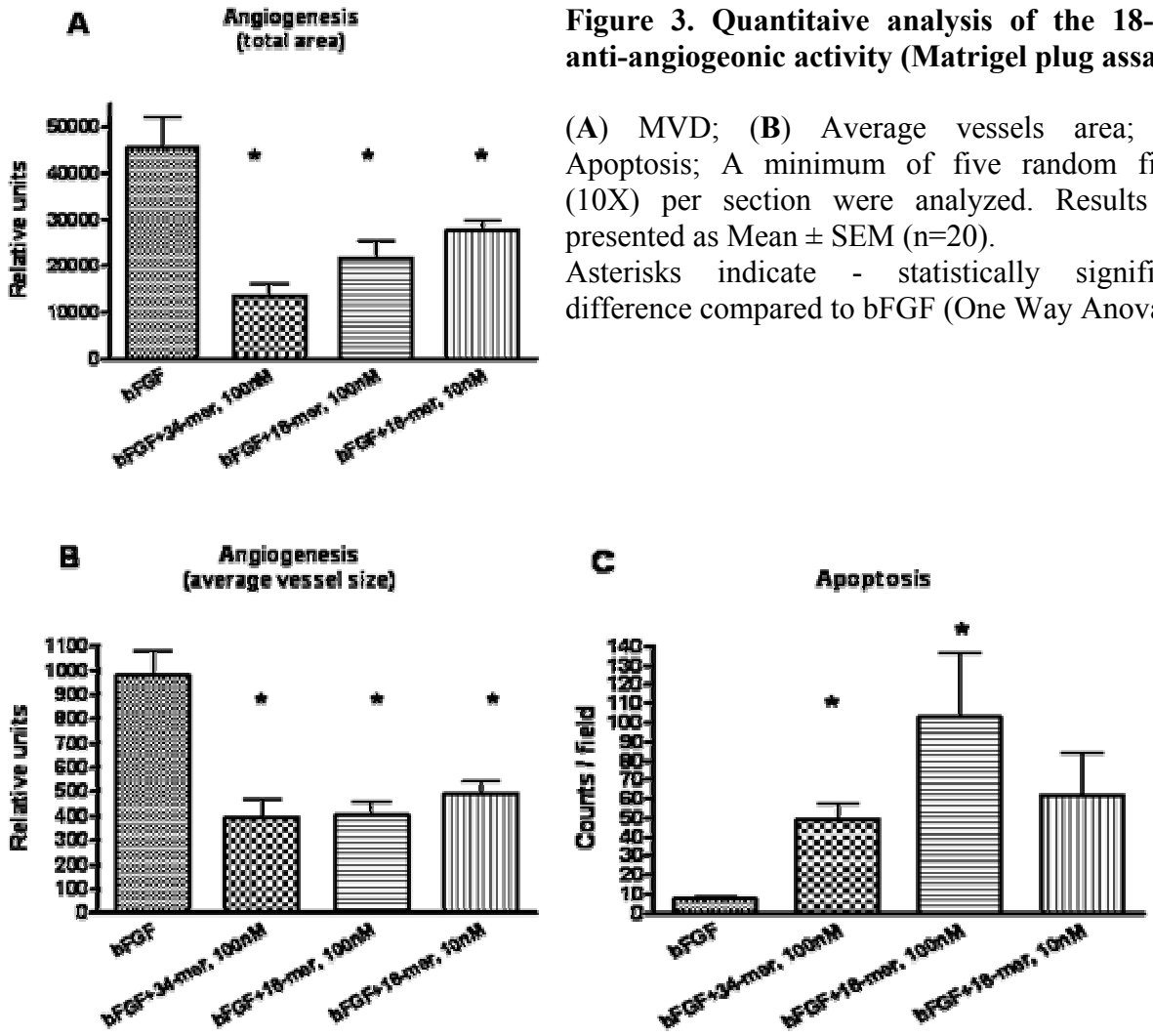

Figure 3. Quantitaive analysis of the 18-mer anti-angiogeonic activity (Matrigel plug assay).

(**A**) MVD; (**B**) Average vessels area; (**C**) Apoptosis; A minimum of five random fields (10X) per section were analyzed. Results are presented as Mean ± SEM (n=20).
Asterisks indicate - statistically significant difference compared to bFGF (One Way Anova).

Both PEDF peptides (the 34-mer and 18-mer) effectively inhibited bFGF-induced angiogenesis in Matrigel plug assay. As shown in Fig. 2 (left panel) and in Fig. 3A, both peptides reduced vascularization (total area covered by vessels). The average size of the vessel was reduced as well in all groups that received peptide treatment (Fig. 2B). Both peptides induced comparable increases in apoptosis (Fig. 1, middle panel and Fig. 2C). At 100 nM the18-mer peptide was more effective at inducing apoptosis then the 34-mer: although the differences were not statistically significant the trend was clear in all assays. The18-mer blocked angiogenesis in a dose-dependent manner: at 10 nM MVD and average vessel size were higher than at 100 nm, while apoptosis was lower (Fig. 2A, B). Overall, Matrigel plug assay demonstrated that PEDF peptides effectively inhibited bFGF-dependent angiogenesis by inducing EC apoptosis.

For this part of the project I had to change mice strain because initially projected C57Bl mice produce highly variable results in angiogenesis assays.
Subsequently, I took steps to determine the effect of the 18-mer on tumor growth in mouse xenograft model.

4. I determined the effect of anti-angiogenic peptides on the growth of androgen-independent carcinoma of the prostate and analyzed angiogenesis
Hormone refractory prostate tumors were established by subcutaneous injections of $2x10^6$ PC3 cells in the right flank region of female nude mice. PEDF peptides (the 18- or 34-mer) were administered intra-peritoneally in PBS at 10mg/kg starting one week post-inoculation (tumors were small but readily visible and palpable). Control animals were treated with PBS. Tumor progression was monitored for 25 days, tumor volume was measured every 2-3 days and calculated as Length x $Width^2$ x 0.523. Data from the two independent experiments were combined and analyzed with Repeated Measurements ANOVA.

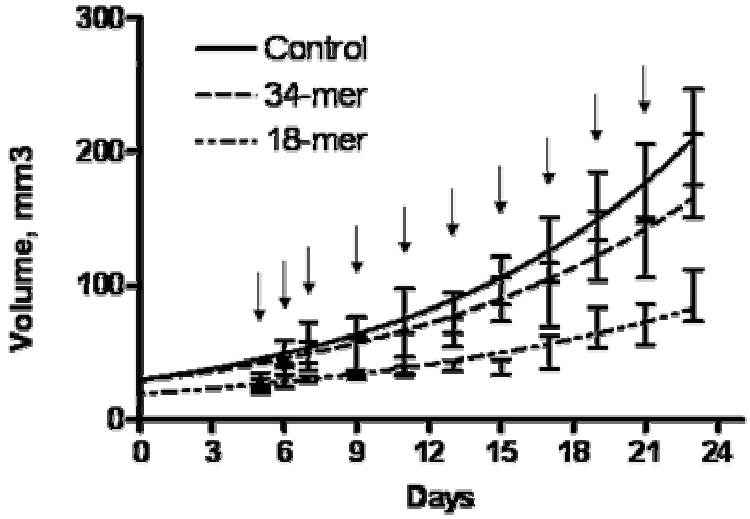

Figure4. The 18-mer inhibits PC-3 tumor growth.
Results are presented as Mean ± SEM (n=8-10) of the 2 independent experiments. Control group received PBS. Peptides were administered at 10 mg/kg. Arrows indicate injection schedule

Under these experimental conditions, the 18-mer inhibited tumor growth (the differences with untreated control and the 34-mer were statistically significant ($p<0.001$ by repeated measures Anova). At the end of the experiment one animal in the 18-mer treated group was completely tumor-free and another had very small tumor (0.523 mm^3). The 34-mer peptide was not effective at the dose tested.

I tested the 18-mer in tumorigenesis assay and stained cryosections of the PC3 tumors to determine the differences in MVD and Apoptosis. To further compare effect of the PEDF peptides on hormone independent prostate cancer, I analyzed frozen sections of harvested tumors for angiogenesis and apoptosis. The sections were stained and analyzed using the procedures described for Matrigel. CD31 EC marker was used to visualize vessels (Fig. 5, left), TUNEL staining to detect apoptosis (Fig 5, middle).
Although the tumor growth curve in animals treated with 34-mer was not significantly different from control, immunofluoresent staining revealed decreased angiogenesis in the 34-mer treated tumors (Fig 5, left) and some increase in apoptosis (Fig 5, middle).

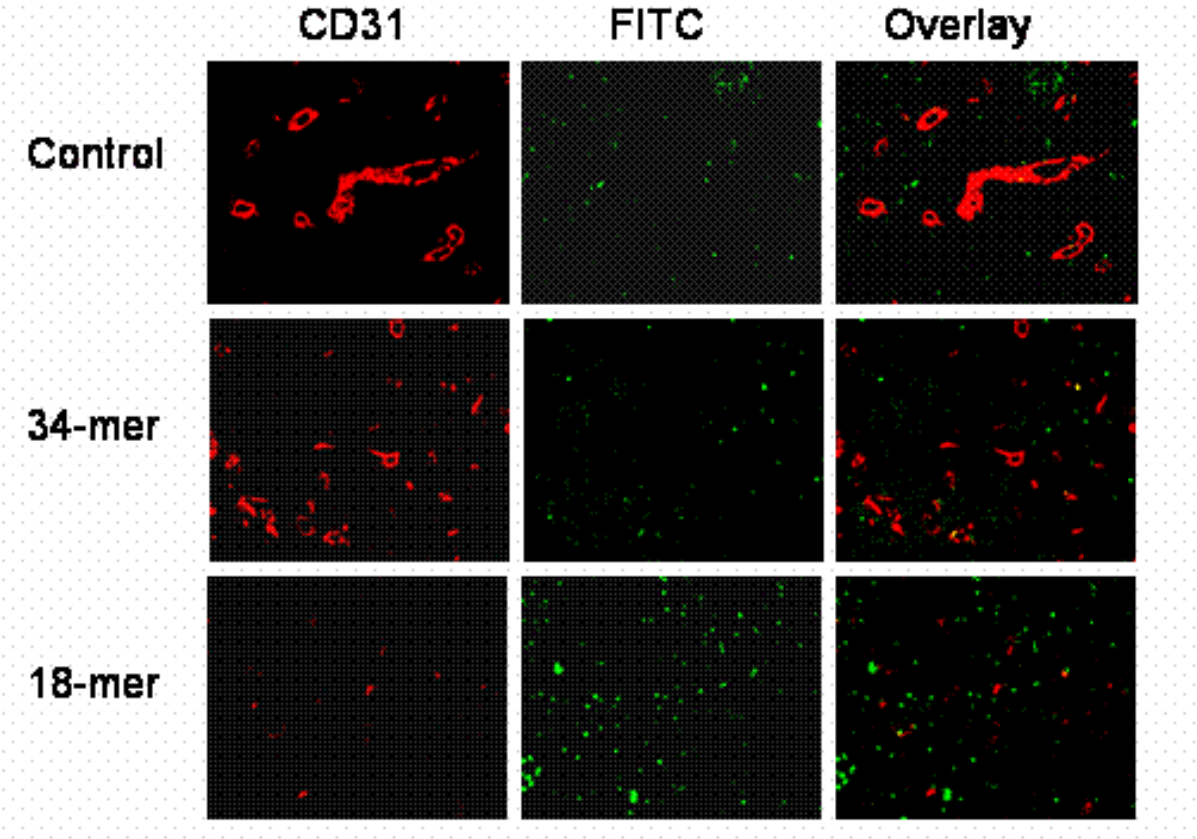

Figure5. Analysis of MVD and apoptosis in PC-3 tumors treated with the 34- and 18-mer. Tumors were harvested, cryosections obtained and stained with fluorescent-labeled antibody for CD31 (red) and ApopTag (green). Overlays are shown on the right. Representative fields are shown (10X magnification).

Quantification of the MVD with MetaMorph software showed statistically significant decreases in tumor angiogenesis (Fig. 6A) in the group treated with either the 34-mer or the 18-mer compared to the sham-treated group ($p < 0.05$ and <0.001, respectively). Average vessel diameter was also decreased in both treatment groups (Fig. 6C) in comparison with control group ($p<0.001$). In addition, apoptosis in these tumors (Fig. 6B) nearly tripled ($p< 0.001$).

While the decrease in the average vessel size was similar in both peptide-treated groups, the reduction of tumor angiogenesis was much more striking in the group treated with the 18-mer (a 3 fold reduction in comparison with sham-treated control). Our results suggest that apoptosis alone may be insufficient to inhibit CaP growth and that dramatic decrease in angiogenesis due to the18-mer treatment may have additional anti-tumor effect.

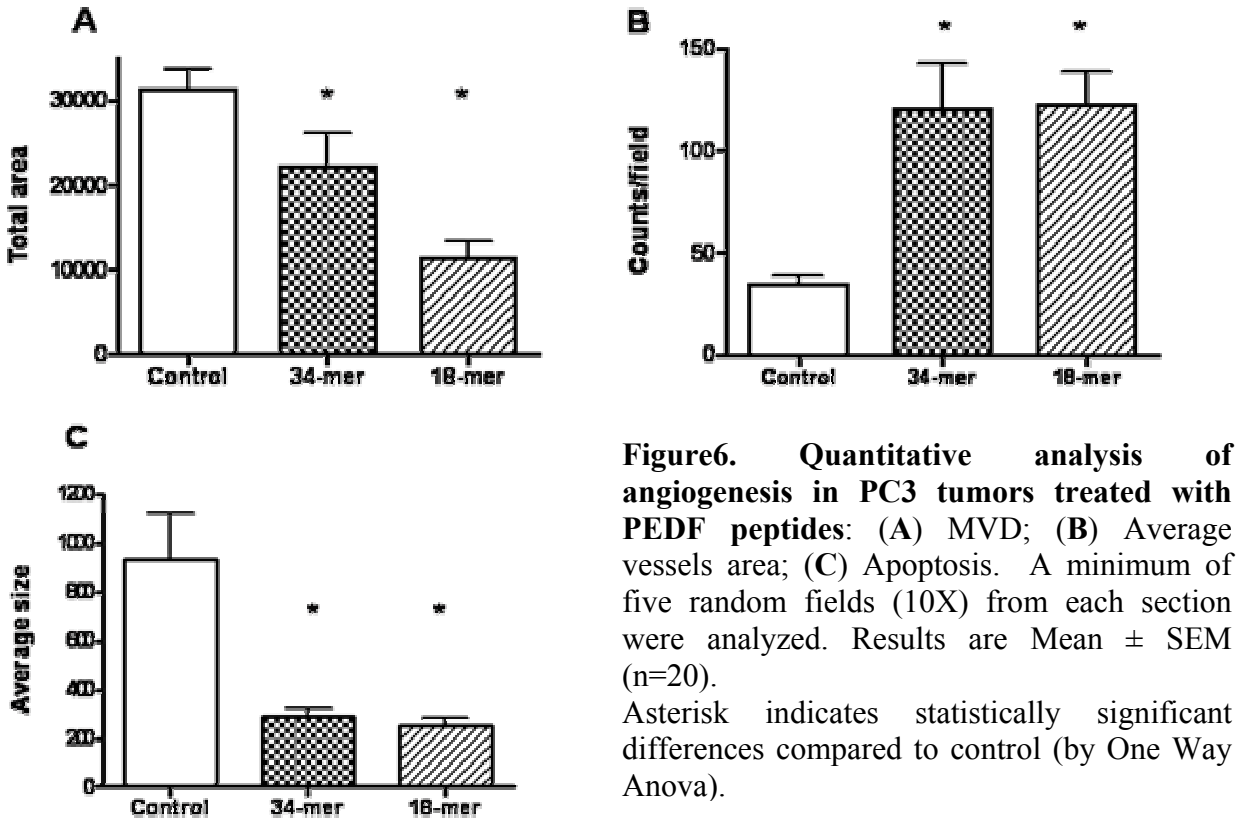

Figure6. Quantitative analysis of angiogenesis in PC3 tumors treated with PEDF peptides: (**A**) MVD; (**B**) Average vessels area; (**C**) Apoptosis. A minimum of five random fields (10X) from each section were analyzed. Results are Mean ± SEM (n=20).
Asterisk indicates statistically significant differences compared to control (by One Way Anova).

A major problem and limitation in the course of this year was unanticipated increases in costs of peptide synthesis and animal procurement. As a result I was unable to test a wider dose range of the 18-mer peptide in the angiogenesis and tumorigenicity assays. However, I consider that I have met the goal of this study to identify the most active anti-angiogenic peptide and confirmed its ability to inhibit prostate cancer growth.

KEY RESEARCH ACCOMPLISHMENTS:

- ✓ In search of the active anti-angiogenic epitope of PEDF, I designed and generated 3 short peptides covering the C-terminal area of its 34-mer anti-angiogenic fragment. These peptides were potential candidates for the anti-angiogenic therapy of prostate cancer.
- ✓ I performed initial screening of the *in vitro* activity of thee peptides and determined the most effective candidate (the18-mer)
- ✓ I have demonstrated the ability of the 18-mer to block neovascularization *in vivo* and compared it to the parental 34-mer and PEDF in corneal micropoket, matrigel plug and DIVAA assays.
- ✓ I have demonstrated the ability of this peptide to inhibit prostate cancer growth in mouse xenograft model and showed that it acts by inhibition angiogenesis and inducing apoptosis in the endothelium and prostate cancer cells. At the dose tested (10 mg/kg/2days) the 18mer activity was superior to that of the parental 34-mer.
- ✓ I recommend this peptide for further testing in tumorigenicity assays to determine optimal dosing and the most efficacious combination treatments.

REPORTABLE OUTCOMES:

Abstract: Mirochnik Y, Filleur S, Volpert O. Pigment Epithelial derived factor: development of anti-angiogenic peptides. 2006. European J Cancer 4S (12):37

Presentation: Symposium on "Molecular Targets and Cancer Therapeutics". Prague, Czech Republic 7-10 November 2006

Abstract: Mirochnik Y, Aurora A., Volpert O. Pigment epithelial derived factor: design and development of anti-angiogenic peptides for the therapy of prostate cancer.

Presentation: IMPaCT Meeting. Atlanta, Georgia USA 5-8 September 2007

US Provisional Patent Application NU27084 "Methods and compositions for inhibiting angiogenesis" Serial# 60/986,124, November 7, 2007

CONCLUSIONS: We designed and tested several peptides, which represent the active epitope of PEDF, a protein with anti-angiogenic, anti-tumor properties. One of the peptides (18-mer) demonstrated striking anti-angiogenic activity *in vitro*. Furthermore, this peptide strongly inhibited prostate tumor growth *in vivo* by blocking angiogenesis and inducing apoptosis in tumor and endothelial cells. Moreover, it was more effective then the parental 34-mer. It was active at low nanomolar concentrations, with negligible toxic effects at higher concentrations and is therefore an excellent candidate to be developed as a novel anti-cancer agent. Although we have demonstrated that inhibition of tumor growth was accompanied by decrease in MVD and increase in apoptosis, further studies are indicted to determine the optimal *in vivo* dosing and effective complementary treatments, stability and the detailed mechanism of the 18-mer action on EC and on cancer cells. In addition, this active peptide may be used in co-crystallization studies with the newly discovered PEDF receptor (*6*) in order to generate small molecule non-protein PEDF mimetics. Such agents are more feasible from the economical and manufacturing standpoint.

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6. L. Notari *et al*., *J Biol Chem* **281**, 38022 (Dec 8, 2006)

APPENDICES:

Appendix – Abstract: Microchnik Y, Filleur S, and Volpert O. “Pigment Epithelial Derived Factor: development of anti-angiogenic peptides”. 2006. European J Cancer 4S (12):37

of treatment) and in one patient at 70 mg (leucopenia grade 2 on 7th week of treatment). The 50 mg were the HMD. Objective antitumor response was documented in 8 among 52 evaluable cases and 32% of patients experienced disease stability for at least 6 months. High pretreatment levels of TSP-1 were associated with objective tumor response (p=. 0003). The steady-state of blood concentrations of vinorelbine (VRL) and 4-O-deacetyl-vinorelbine (DVRL) ranged around 1 ng/ml and were consistent with expected low accumulation.
Conclusions: Protracted administration of metronomic oral vinorelbine is feasible at doses up to 50 mg administered 3 times a week. The observed durable antitumor effects against chemo-resistant tumors at doses lacking of undesirable side effects taken together with pharmacokinetics and featured predictive biomarkers provide clinical evidence supporting that metronomic therapy with vinorelbine primarily targets the vascular network of tumors. A randomized phase II study is now recruiting patients to define the optimal metronomic dose of oral vinorelbine.

110 POSTER

Synergistic effect of nab-paclitaxel and anti-VEGF-A antibody (bevacizumab) against the metastasis of breast tumor xenografts

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Background: *nab*-Paclitaxel (Abraxane®; ABX) is a 130-nm, albumin-bound paclitaxel that has shown greater efficacy and less toxicity than solvent-based paclitaxel in several xenograft models and in clinical trials. This study was designed to determine the effects of ABX and anti-VEGF-A antibody (bevacizumab; Avastin®; AVA), as single or combined therapy, on the growth of orthotopically implanted MDA-MB-231 tumors and on metastatic spread to the lungs and lymph nodes (LNs).
Material and Methods: Luciferase-expressing MDA-MB-231 human breast carcinoma cells were implanted into mammary fatpads of female *nu*/*nu* mice and allowed to reach an average size of 230 mm^3 before treatment. Ten mice were treated with 1 or 2 cycles of ABX (10 mg/kg, qdx5), followed by injection of AVA (4 mg/kg, 2/wkx6). Additional groups received ABX alone, AVA alone, or saline. Mice were monitored for tumor growth and toxicity. Mice were sacrificed when mean tumor volume in the saline-treated group reached 2000 mm^3. Luciferase activity was measured in extracts prepared from the 10 axillary LNs and both lobes of the lungs of each mouse.
Results: No toxicity was observed in any group. Tumors reached an average size of 1000 mm^3/group on days 25, 30, 45, and 80 after treatment with saline, AVA, and 1 and 2 cycles of ABX, respectively. Combined AVA and ABX therapy, particularly with 2 cycles of ABX, yielded a significantly better outcome than either therapy alone (30% of mice had complete regression; tumors in the remaining mice were reduced by 90% compared with controls). Only the combined therapy reduced metastasis to the lungs and LNs, with 6 of the 20 mice in combination therapy having no metastases to lungs or LNs ($P = 0.03$ vs controls, Fisher exact test). Total metastatic burden to LNs was reduced in a dose-dependent manner, with 42%, 85%, and 82% suppression of LN metastasis burden at AVA doses of 2, 4, and 8 mg/kg, respectively. AVA alone suppressed LN metastasis by only 8%. Metastatic burden to the lungs was not sufficient for statistical analysis, although the same trend was observed.
Conclusions: As expected, AVA alone did not significantly inhibit primary tumors or metastasis. The efficacy of ABX was much higher than that of AVA and was substantially improved by adding a second cycle of the drug. However, only the combination of ABX and AVA eradicated primary tumors in 30% of the mice and completely eliminated regional and distant metastases in 70% of the treated animals.

111 POSTER

A dose escalation study of AMG 386, a selective inhibitor of angiopoietin-2, in adult patients with advanced solid tumors

R. Herbst[1], R. Kurzrock[2], C. Storgard[3], E. Malseed[3], L. Rosen[4]. *[1]The University of Texas M. D. Anderson Cancer Cent, Thoracic/Head and Neck Medical Oncology, Houston, TX, USA; [2]The University of Texas M. D. Anderson Cancer Cent, Experimental Therapeutics, Houston, TX, USA; [3]Amgen Inc., Thousand Oaks, CA, USA; [4]Premiere Oncology, Santa Monica, CA, USA*

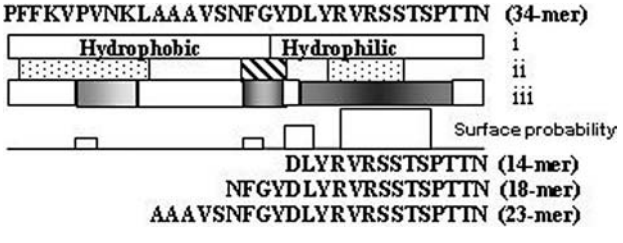
Background: Angiopoietin-2, is upregulated at sites of tumor angiogenesis, and promotes new vessel growth through interaction with its receptor, Tie2. AMG 386 is a peptide-Fc fusion protein (peptibody) that inhibits the interaction between angiopoietins and Tie2. In preclinical tumor models, AMG 386 treatment results in decreased endothelial proliferation, increased tumor necrosis and decreased tumor growth, supporting further evaluation of AMG 386 as a novel therapeutic in Phase 1 cancer trials either alone or in combination.
The objectives of this first in human Phase 1 study are to assess the safety and pharmacokinetics (PK) of AMG 386 in adult subjects with advanced solid tumors.
Methods: Adult subjects were sequentially enrolled into 5 dose cohorts and received weekly intravenous doses of AMG 386 at 0.3, 1, 3, 10, and 30 mg/kg. Safety assessments included adverse events (AEs), clinical laboratories, vital signs, ECG monitoring, and anti-AMG 386 antibody formation. Tumor response was also assessed.
Results: 22 subjects have been treated in this dose escalation Phase 1 study with safety data available to date for 21 [(10 M/12 F); median age (range): 55 (43–79)]. Tumor types included: non-small cell lung, pancreatic, colorectal, hemangio, sarcoma, ovarian, breast, thyroid, renal, pseudomyxoma, parotid, and adenocarcinoma of unknown primary origin. Treatment-related AEs were generally mild or moderate (most CTCAE grade 1 or 2), with only fatigue (n = 7), gastrointestinal disorder (n = 3), and peripheral edema (n = 2) reported in more than 1 pt. One dose limiting toxicity (DLT) at 30 mg/kg was observed. Dose-linear PK was observed and the half-life supports weekly dosing. Serum concentrations reached steady state by week 3. Minimal accumulation was observed after multiple doses. Neutralizing antibodies were not detected. Best RECIST responses include stable disease (n = 16, 76%) and progressive disease (n = 5, 24%).
Conclusions: Weekly administration of AMG 386 up to 30 mg/kg was safe and well tolerated. The maximum tolerated dose was not reached. Minor AEs do not appear to be dose-related; 1 DLT was observed. 76% of subjects experienced stable disease (SD). The observation of a significant number of patients with SD is encouraging and supports evaluation of AMG 386 in further clinical studies.

112 POSTER

Pigment Epithelial-Derived Factor: development of anti-angiogenic peptides

Y. Mirochnik, S. Filleur, O. Volpert. *Northwestern University, Feinberg Medical School, Chicago, USA*

Background: Pigment epithelial-derived factor (PEDF) is a potent natural angiogenesis inhibitor. We have recently mapped PEDF anti-angiogenic activity to its 34-mer N terminal peptide (residues 24−57). This peptide retains PEDF anti-angiogenic properties: it reproduces signaling events in endothelial cell (EC), elicits EC apoptosis and blocks migration. Forced expression of 34-mer peptide delays the growth of prostate carcinoma. In this study we designed and screened short synthetic PEDF peptides for potential use as anti-angiogenic/anti-cancer therapeutics.
Material and Methods: The 3D structure of the 34-mer peptide was analyzed using Protean software in terms of relative hydrophobicity, charge distribution, and antigenic index. Short synthetic peptides covering the 34-mer fragment were generated (see figure) and tested for the ability to cause apoptosis and inhibit EC migration. Peptides were further tested for anti-angiogenic activity *in vivo* in matrigel plug and mouse corneal assays.



Results: The 34-mer C-terminus is strongly hydrophilic, with highly charged central area and high antigenic index, and is likely to interact with a target receptor. All screened peptides (14, 18 and 23-mer) demonstrated activity in EC apoptosis and migration assays. Dose-response curves were generated and the potency of the peptides compared to native PEDF and the 34-mer. Although all peptides showed anti-angiogenic activity *in vitro*, only one remained active *in vivo* due to stability differences. Neither of the peptides showed signs of toxicity at the doses tested.
Conclusion: We generated short peptides that reproduce the anti-angiogenic activity of PEDF in vitro and in vivo. These peptides will be tested in pre-clinical models of prostate cancer and melanoma and, if active, proposed for early stage clinical trials.